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RESEARCH ARTICLE

The effect of an isoflavonid-rich liquorice extract on fermentation, methanogenesis and the microbiome in the rumen simulation technique

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One sentence summary: An isoflavonoid-rich liquorice extract could potentially be used to boost productivity and decrease the environmental burden of livestock production due to its effects on rumen microbial communities.
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ABSTRACT

Due to the antimicrobial activity of flavonoids, it has been suggested that they may provide a possible alternative to antibiotics to stimulate productivity and reduce the environmental load of ruminant agriculture. We hypothesised that an extract of liquorice, rich in prenylated isoflavonoids and particularly glabridin, might potentially improve the efficiency of nitrogen utilisation and reduce methane production in the rumen. When added to a long-term rumen simulating fermentor (RUSITEC), liquorice extract at 1 g L^{-1} decreased ammonia production (−51%; $P < 0.001$) without affecting the overall fermentation process. When added at 2 g L^{-1}, decreases in not only ammonia production (−77%; $P < 0.001$), but also methane (−27%; $P = 0.039$) and total VFA production (−15%; $P = 0.003$) were observed. These effects in fermentation were probably related to a decrease in protozoa numbers, a less diverse bacteria population as well as changes in the structure of both the bacterial and archaeal communities. The inclusion of an isoflavonoid-rich extract from liquorice in the diet may potentially improve the efficiency of the feed utilisation by ruminants.

Keywords: glabridin; isoflavonoids; liquorice; methane; rumen fermentation

INTRODUCTION

Since the ban of antibiotics as growth-promoting feed additives by the European Union in 2006, plant extracts and plant secondary metabolites have been considered as alternatives to manipulate rumen fermentation to boost productivity and decrease the environmental burden of livestock production (Hart *et al*. 2008). Among plant secondary metabolites, flavonoids have recently gained interest because of their wide range of biological activities, particularly antimicrobial properties (Oskoueian, Abdullah and Oskoueian 2013).

Flavonoids are polyphenolic compounds consisting of a 15-carbon skeleton in which two benzene rings are linked via a heterocyclic pyran ring (Kumar and Pandey 2013). According to substitution pattern variations, flavonoids can be classified into

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different subclasses, providing an extremely diverse range of derivatives (Wang, Li and Bi 2018). Depending on their chemical structure, flavonoids can then have different antimicrobial effects (Wang, Li and Bi 2018) that ultimately determine the extent in which rumen fermentation can be altered (Oskoueian, Abdullah and Oskoueian 2013).

The effect of flavonoids on rumen fermentation has not been extensively evaluated (Patra *et al.* 2017). In addition and taking into account their great variability in structure (over 9000 different compounds identified; Wang, Li and Bi 2018), only a small number of flavonoid-rich plant extracts or pure compounds have been tested so far. Some flavonoids, or the derivatives produced by microbial degradation in the rumen, have been reported to affect rumen microbial activity causing, amongst other effects, a decrease in methane production (Oskoueian, Abdullah and Oskoueian 2013; Kim *et al.* 2015; Ma *et al.* 2017). Furthermore, flavonoids have been shown to be effective in attenuating the effects of excessive grain feeding on rumen pH (Balcells *et al.* 2012; de Nardi *et al.* 2014). However, to our knowledge, a detailed characterisation of the changes in rumen microbial communities associated with the effects of flavonoids on rumen fermentation has not yet been published. In this study, we tested an extract of liquorice, rich in prenylated isoflavonoids and particularly glabridin (Asl and Hosseinzadeh 2008), for its effect in *in vitro* batch culture and its long-term effect on rumen fermentation and methanogenesis whilst also characterising its effect on bacterial and methanogen communities.

MATERIAL AND METHODS

Liquorice extract

Liquorice extract was obtained from the dried roots of liquorice (*Glycyrrhiza glabra*; 40 g) after extraction with 95% ethanol (2 L for 2 h) at 45°C and then concentrating under reduced pressure to give a 95% ethanol extract (1.20 g). The extract was further purified on silica gel eluted with ethyl acetate–methanol gradients (19 : 1; 9 : 1; 2 : 1) and finally with methanol. Nuclear magnetic resonance analysis revealed that glabridin was the major flavonoid in the extract, with five other related flavonoids found. Fitness R Us Ltd (Kiryat Shmona, Israel) provided the extract as Licogen powder (Batch No: 14090023PWDR). Liquorice powder is sold as a natural antioxidant, flavouring powder and phytoestrogen for menopausal women.

Measurement of protozoal activity

The effect of liquorice extract on protozoal activity was measured *in vitro* as the breakdown of [^{14}C]-labelled bacteria by rumen protozoa as described by Wallace and McPherson (1987). Isotope-labelled bacteria were obtained by growing *Streptococcus bovis* ES1 in Wallace and McPherson media (Wallace and McPherson 1987) containing [^{14}C] leucine (1.89 μCi/7.5 mL tube) as the sole nitrogen source, for 24 h. Cultures were centrifuged (3000 g, 15 min), supernatant discarded and pellets re-suspended in 7 mL of simplex-type salt solution (STS; Williams and Coleman 1992) containing ^{12}C-leucine (5 mM). This process was repeated three times to prevent re-incorporation of released [^{14}C] leucine by bacteria.

Rumen digesta was obtained from four rumen-cannulated Holstein–Frisian cows fed at maintenance level (composed of perennial ryegrass hay and concentrate at 67 : 33 on a DM basis). Animal procedures were carried out in accordance with the Animal Scientific Procedures Act 1986, and protocols were approved by the Aberystwyth University Ethical Committee. Rumen digesta was obtained before the morning feeding and strained through two layers of muslin and diluted with STS (1 : 1) containing ^{12}C-leucine (5 mM). Diluted rumen fluid (7.5 mL) was then incubated with labelled bacteria prepared as described above (0.5 mL) in tubes containing no additive (control) or 0.25, 0.5, 1 or 2 g L^{-1} of liquorice extract. Incubations were carried out at 39°C under a stream of CO_2, and tubes were sampled at time 0 and at 1 h intervals up to 5 h using a syringe with a 19 gauge needle. Samples (0.5 mL) were acidified (by adding 0.125 mL of 25% trichloroacetic acid (wt/vol) and centrifuged (13 000 *g*, 5 min). Supernatant (0.200 mL) was diluted with 2 mL of OptiPhase HiSafe 2 scintillation fluid (Perkin Elmer, Seer Green, UK) to determine the radioactivity released by liquid-scintillation spectrometry (Hidex 300 SL, Lablogic Systems Ltd, Broomhill, UK). Bacterial breakdown at each incubation time was expressed as the percentage of the acid-soluble radioactivity released relative to the total radioactivity present in the initial labelled bacteria (Wallace and McPherson 1987).

In vitro batch cultures

To measure the short term effect of liquorice extract on fermentation parameters, 24 h *in vitro* incubations were carried out. The experimental design consisted of a control (no additive) and liquorice extract added at 0.5, 1 or 2 g L^{-1}. The experiment was conducted in quadruplicate, using rumen fluid from the same four cannulated cows. Rumen contents were sampled before the morning feeding, filtered through a double layer of muslin and diluted 1 : 2 in artificial saliva solution (Menke and Steingass 1988). Aliquots (30 mL) of the diluted strained rumen fluid were added anaerobically to 120 mL Wheaton bottles containing 0.3 g of diet composed of ryegrass hay and barley (40 : 60), previously ground to pass through a 1-mm^2 mesh screen. Bottles were sealed and incubated at 39°C receiving a gentle mix before sampling at 24 h.

Fermentation pattern, in terms of pH, ammonia and VFA was determined after 24 h of the incubation. A subsample (4 mL) was diluted with 1 mL of deproteinising solution (200 mL L^{-1} orthophosphoric acid containing 20 mmol L^{-1} of 2-ethylbutyric acid as an internal standard) for the determination of VFA using gas chromatography, as described by Stewart and Duncan (1985). Another subsample (1 mL) was diluted with 0.250 mL of 25% trichloroacetic acid (wt/vol) for analysis of ammonia using a colourimetric method (Weatherburn 1967).

Rumen simulation technique

The rumen simulation technique (RUSITEC; Czerkawski and Breckenridge 1977) was used to study the effect of a control diet alone or supplemented with liquorice extract at 1 g L^{-1} (liquorice 1; 0.66 g d^{-1}, 3.3% inclusion rate in DM) or 2 g L^{-1} (liquorice 2; 1.32 g d^{-1}, 6.6% inclusion rate in DM), doses that were selected based on the results obtained in the 24 h batch culture trial described above. The experimental diet was the same one used in the batch culture trial (40 : 60, ryegrass hay and barley grounded to pass through 1 mm^2 sieve size).

Rumen digesta was obtained from four rumen-cannulated Aberdale x Texel sheep, fed at maintenance level (diet composed of perennial ryegrass hay and concentrate at 67 : 33 on DM basis). Animal procedures were carried out in accordance with the Animal Scientific Procedures Act 1986, and protocols were approved by the Aberystwyth University Ethical Committee. Rumen digesta was obtained before the morning feeding,

strained through two layers of muslin and stored anaerobically at 39°C.

The trial consisted of a single incubation period using 12 vessels that were considered as experimental units. Each dietary treatment was randomly allocated to the vessels that were inoculated with rumen fluid from four different sheep (four replicates). Vessels had an effective volume of 800 mL and were kept at 39°C under permanent vertical agitation.

On day 1, vessels were inoculated with strained rumen fluid mixed with artificial saliva (McDougall 1948) and demineralised water in a 1 : 1 : 1 ratio. Then, artificial saliva was continuously infused at a rate of 660 mL d^{-1} (dilution rate of 3%/h) using a multichannel peristaltic pump (Watson–Marlow 200 series, Cornwall, UK). Squeezed rumen solids (20 g FM) were placed in nylon bags (110 × 60 mm, pore size 100 μm^2) and incubated in each vessel for 1 day to provide solid-associated bacteria, while experimental feed (20 g DM) was supplied in a second bag. On subsequent days, the feed bag that had remained 2 days in each vessel was squeezed, returning the liquid to the vessel, and discarded; a new bag, containing 20 g DM was then inserted to the vessel.

The trial lasted for 18 days, using the first 12 days for adaptation and the last 6 for sampling. Dry matter degradation, total gas and methane production and outflow of fermentation products were measured on days 13, 14, 15 and 16. Nylon bags were collected, rinsed with cold water for 20 min, and DM disappearance after 48 h incubation was calculated from the loss in weight. The residue was then analysed for organic matter (OM), nitrogen (N), Neutral-detergent (NDF) and Acid-detergent fibre (ADF) to determine nutrient disappearance. Fermentation gases were collected in gas-tight bags (TECOBAG 5L, PETP/AL/PE-12/12/75, Tesseraux container GmbH, Germany) to measure total gas and methane production. Daily production of ammonia and VFA were measured in the overflow flasks with 10 mL of saturated $HgCl_2$ (diluted 1 : 5) added to stop the fermentation.

To describe diurnal changes in the fermentation pattern, on days 17 and 18 the content of the vessels was sampled (25 mL) by aspiration at 0, 2, 4 and 8 h after feeding. The pH was immediately recorded, and five subsamples were collected as follows: for microbial characterisation and enzymatic activity, 16 mL were collected and immediately frozen in liquid N prior to long-term storage at −80°C. For VFA determination, 1.6 mL of sample was diluted with 0.4 mL of deproteinising solution (200 mL L^{-1} orthophosphoric acid containing 20 mmol L^{-1} of 2-ethylbutyric acid as an internal standard). For ammonia analysis, 0.8 mL of sample was diluted with 0.2 mL of trichloroacetate (25% wt:vol). For lactate determination, 1 mL sample was collected and snapped frozen in liquid N prior to long-term storage at −80°C. For protozoa counts, 0.5 mL of sample were added to 0.5 mL of saline formaline solution (4% formaldehyde and 0.9% NaCl in distilled water) and stored at room tempreture.

Sample analyses

For feed analysis, DM and OM content were determined by drying in an oven at 105°C for 24 h and heating at 550°C for 6 h in a muffle furnace, respectively. Nitrogen concentration was measured by the Dumas combustion method (Elementar analyser, Vario MAX cube, Hanau, Germany). For NDF and ADF determination, the automated fibre analyser (ANKOM 2000, Macedon, USA) was used. Methane concentration was determined by directly injecting 0.5 mL of gas sample into a gas chromatograph (ATI Unicam 610 Series, Cambridge, UK) fitted with a 40 cm Porapak N metal packed column (Agilent, Cheshire, UK) and flame ionisation detector. Ammonia and VFA concentrations in vessels and overflows were determined as described by Weatherburn (1967) using an automated spectrophotometer (ChemWell T,Astoria Pacific, Oregon, USA) and Stewart and Duncan (1985) using gas chromatography, respectively. Protozoa were quantified by optical microscope following the procedure described by Dehority (1993) and adapted by de la Fuente, Skirnisson and Dehority (2006). Concentrations of L-lactate and D-Lactate were measured using the Enzytec D/L-Lactic Acid kit (r-biopharm, Darmstadt, Germany); total lactate was calculated as the sum of both. Enzymatic activities in vessels content were measured according to the procedure described by Giraldo *et al.* (2008) and Belanche at al. (2016). Endoglucanase (EC 3.2.1.4.), xylanase (EC 3.2.1.8.) and amylase activities (EC 3.2.1.1.) were measured in triplicate and expressed as mmol of sugar released from the corresponding substrates in 1 min per gram DM of sample (or gram of protein).

DNA extraction and quantitative PCR

Genomic DNA was extracted from vessel samples withdrawn at different time points. Freeze-dried samples (25 mg DM) were bead beaten in 4% SDS lysis buffer for 45 s, and DNA was extracted using a CTAB/Chloroform method (adapted from Yu and Morrison 2004).

Concentration and quality of genomic DNA was assessed by spectrophotometry (Nanodrop ND-100, Thermo Scientific, USA). Absolute concentrations of DNA from total bacteria, methanogens and fungi were determined by qPCR and serial dilutions of their respective standards (10^{-1}–10^{-5}) as previously described (Belanche *et al.* 2012, 2016). Quantitative PCR (qPCR) was conducted in triplicate using a LightCycler 480 System (Roche, Mannheim, Germany).

Ion torrent next generation sequencing

Rumen bacteria and methanogenic archaea communities were studied using Next Generation Sequencing (NGS) (de la Fuente *et al.* 2014). For bacterial profiling, amplification of the V1–V2 hypervariable regions of the 16S rRNA gene was carried out using bacterial primers (27F and 357R) followed by Ion Torrent adaptors. For methanogens profiling, amplification of the V2-V3 hypervariable region of the 16S rRNA gene was performed using archaeal primers (86F and 519R) also followed by Ion Torrent adaptors.

Forward primers were barcoded with 10 nucleotides to allow sample identification. PCR was carried out on a 25 μL reaction containing DNA template (1 μL), 0.2 μL reverse primer, 1 μL forward primer, 5 μL buffer (PCR Biosystems Ltd., London, UK), 0.25 μL bio HiFi polymerase (PCR Biosystems) and 17.6 μL molecular grade water. Amplification conditions for bacteria and methanogens were 95°C for 1 min, and then 22 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 30 s. To assess quality of amplifications, resultant amplicons were visualised on a 1% agarose gel. PCR products were then purified using Agencourt AMpure XP beads (Beckman Coulter Inc., Fullerton, USA), and DNA concentration was determined using an Epoch Microplate Spectrophotometer fitted with a Take 3 Micro-Volume plate (BioTek, Potton, UK) to enable equimolar pooling of samples with unique barcodes.

Libraries were further purified using the EGel system with 2% agarose gel (Life Technologies Ltd., Paisley, UK). Purified libraries were assessed for quality and quantified on an Agilent 2100 Bioanalyzer with High Sensitivity DNA chip (Agilent Technologies Ltd., Stockport, UK). Library preparation for NGS

sequencing was carried out using the Ion Chef system (Life Technologies UK Ltd) and the Ion PGM HiQ Chef kit, and sequencing using the Ion Torrent Personal Genome Machine (PGM) system on an Ion PGM Sequencing 316 Chip v2 BC. Due to the lower abundance of methanogens than total bacteria, methanogens library was sequenced using a smaller chip (Ion PGM Sequencing 314 Chip v2).

Following sequencing, data were processed as previously described (de la Fuente *et al*. 2014). Briefly, sample identification numbers were assigned to multiplexed reads using the MOTHUR software package. Data were denoised by removing low-quality sequences, sequencing errors and chimeras (quality parameters: maximum 10 homopolymers, qaverage 13, qwindow 25, for archaea the qwindow was set at 30, and erate = 1; Chimera check, both de novo and database driven using Uchime).

Sequences were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II (Wang *et al* 2007), while the methanogens were compared with the RIM-DB database (Seedorf *et al*. 2014). The number of reads per sample was normalised to the sample with the lowest number of sequences. To exclude potential bacterial sequences from the methanogens dataset, methanogens sequences were blasted with the Ribosomal Database Project-II, and those annotations that matched with bacterial sequences were removed. Raw sequences reads from the bacterial and methanogens libraries were deposited at the EBI Short Read Archive of the European Nucleotide Archive (accession number PRJEB22945 and PRJEB22960, respectively).

Statistical analysis

Linear regression was conducted to model the relationship between the percentage of radioactivity released (relative to the ^{14}C-bacterial inoculum) and the time (from 0 h to 5 h), as well as its correlation coefficient. The slope of this trend-line indicated the bacterial degradation rate (as % h^{-1}) by the rumen protozoa and ultimately their activity. Trend line slopes, 24 h fermentation parameters, daily productions of VFA and ammonia in the RUSITEC system together with nutrient disappearance and methane data were analysed statistically by randomised block ANOVA, with individual cows/sheep as a blocking term. For the rates of bacterial degradation and 24 h fermentation parameters, polynomial contrasts were also used to determine linear (L) and/or quadratic (Q) responses to the treatments. Rumen fermentation and qPCR data in the RUSITEC were analysed using a repeated-measurements procedure (REML) including the different time-points (0, 2, 4 and 8). The effect of treatment, time and treatment × time interaction on the relative abundance of different bacteria, and archaea taxa was analysed by split plot ANOVA (three treatments × four time points). *P* values were adjusted for multiple testing using the method proposed by Benjamini and Hochberg (1995) to decrease the false discovery rate. When effects were detected, treatment means were compared by Fisher's protected LSD test. Findings with $P < 0.05$, $P < 0.10$ when applying Benjamini and Hochberg (1995) correction, were regarded statistically significant. Genstat 15th Edition (VSN International, Hemel Hempstead, UK) was used.

Permutation multivariate analysis of variance (PERMANOVA) was used to determine overall significant differences in bacterial and archaea community and was performed in PRIMER 6 and PERMANOVA + (versions 6.1.18 and 1.0.8, respectively; Primer-E, Ivybridge, UK). Abundance percentage data were subjected to square root transformation and Bray–Curtis distance matrices calculated. PERMANOVA was carried out using default settings with 9999 unrestricted permutations, and the Monte Carlo *P* value was calculated. Analysis of Similarity (ANOSIM) was carried out in PRIMER 6 and PERMANOVA + using the Bray–Curtis distance matrix calculated above. This analysis was used to provide a metric of the degree of divergence between communities as given by the R statistic.

To calculate the contribution of environmental data on bacteria and archaea communities, distance-based linear modelling was used to calculate which environmental variables had a significant correlation with the community data. Significant variables were used in distance-based redundancy analysis (dbRDA) (Legendre and Anderson 1999) as implemented in PRIMER 6 and PERMANOVA+.

Table 1. Effect of liquorice extract at 0.5, 1 and 2 g L^{-1} on pH, NH_3-N and VFA profile in ruminal digesta after 24 h of incubation.

	Dose (g L^{-1})						
	0	0.5	1	2	SED	P	Contrast
pH	6.44^{b}	6.44^{b}	6.37^{a}	6.35^{a}	0.017	0.001	L***
NH3-N (mg L^{-1})	20.5^{c}	19.5^{bc}	18.2^{ab}	16.2^{a}	1.00	0.010	L**
Total VFAs (mmo L^{-1})	77.5	77.7	79.4	77.9	4.10	0.965	–
Molar proportions							
Acetate	59.2	59.0	59.6	57.1	3.14	0.858	–
Propionate	17.4^{a}	18.0^{ab}	19.8^{b}	22.9^{c}	1.03	0.002	L***
Butyrate	12.0^{b}	11.8^{b}	11.4^{b}	9.42^{a}	0.666	0.013	L**
BCVFA	2.49^{b}	2.42^{b}	2.34^{b}	1.95^{a}	0.149	0.024	L**

$^{a–c}$Means with different superscript differ ($P < 0.05$); L: linear response; **$P < 0.01$; ***$P < 0.001$. BCVFA = Branched chain volatile fatty acids.

RESULTS

Acute antiprotozoal activity and effect on fermentation parameters (*in vitro* batch incubations)

Bacterial degradation by protozoa increased linearly ($R^2 > 0.99$) over the 5 h incubation with the control treatment. Increasing levels of liquorice extract resulted in a linear and quadratic decrease ($P < 0.001$) in the breakdown of bacteria by protozoa (Table S1, Supporting Information). Whereas the rate of bacterial breakdown was not affected by the addition of 0.25 g L^{-1} of the flavonoid-rich extract, it was reduced by 55.6% ($P < 0.001$) in the presence of 0.5 g L^{-1}. Doses of 1 and 2 g L^{-1} of liquorice extract caused a dramatic reduction in protozoa activity ($P < 0.001$) with no bacterial breakdown observed.

Based on these results, doses of 0.5, 1 and 2 g L^{-1} of the extract were tested over 24 h in *in vitro* incubations (Table 1). Liquorice extract added at 1 and 2 g L^{-1} of the incubation caused only a moderate decrease in pH, albeit significant ($P = 0.001$), compared with the control. No effect on the concentration of total VFA or on the molar proportion of acetate was observed at any of the concentrations tested ($P > 0.05$). Doses of 1 and 2 g L^{-1} resulted in a decrease in ammonia concentration ($P = 0.010$) and in an increase in the molar proportion of propionate ($P = 0.002$). A reduction in the molar proportions of butyrate ($P = 0.013$) and branched chain volatile fatty acids (BCVFA) ($P = 0.024$) was only observed with the highest dose of liquorice.

Table 2. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g L^{-1}, respectively) on feed disappearance in the RUSITEC system.

Treatment	C	L1	L2	SED	P
Disappearance (%)					
DM	43.4	43.4	42.2	1.42	0.653
OM	47.2	45.4	42.3	1.67	0.069
N	43.2	43.3	43.3	1.77	0.998
NDF	38.7	37.0	37.2	1.01	0.257
ADF	44.2	43.5	43.2	0.76	0.451

Table 3. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g L^{-1}, respectively) on fermentation products and methanogenesis in the RUSITEC system.

Treatment	C	L1	L2	SED	P
Fermentation products (mmol d^{-1})					
Total VFA	33.7^{b}	34.1^{b}	28.5^{a}	1.056	0.003
Acetate	17.2^{b}	17.0^{b}	12.8^{a}	0.795	0.003
Propionate	3.55^{a}	4.06^{a}	4.94^{b}	0.313	0.012
Butyrate	8.08	7.95	7.56	0.333	0.332
BCVFA	3.51^{b}	3.74^{b}	0.482^{a}	0.213	<0.001
Ammonia	1.37^{c}	0.674^{b}	0.315^{a}	0.106	<0.001
Gas emissions					
Total gas (L d^{-1})	1.20	1.23	1.30	0.069	0.384
Methane (mM)	3.92^{b}	3.58^{b}	2.37^{a}	0.175	<0.001
Methane (mmol d^{-1})	4.67^{b}	4.35^{b}	3.06^{a}	0.445	0.024
Methane (mmol $gDOM^{-1}$)	0.510^{b}	0.492^{b}	0.374^{a}	0.043	0.039
2H produced (mmol d^{-1})	70.2^{b}	69.8^{b}	60.8^{a}	1.59	0.002

^{a} and ^{b}Means with different superscript differ ($P < 0.05$). BCVFA = Branched chain volatile fatty acids.

Feed degradability and fermentation pattern (rumen simulation technique)

Because the batch culture experiment showed no effect on fermentation with liquorice added at 0.5 g L^{-1}, only doses of 1 and 2 g L^{-1} were further tested in the RUSITEC system.

The addition of liquorice to the diet did not have any detrimental effect on feed disappearance after 48 h of incubation (Table 2), although a trend ($P = 0.069$) to decreased OM disappearance was observed with the highest dose of liquorice tested. When liquorice was added at 1 g L^{-1}, no negative effects on fermentation were observed, whilst ammonia production decreased (−51%; $P < 0.001$). The addition of 2 g L^{-1}, however, had a strong effect decreasing total VFA concentration ($P = 0.014$), shifting fermentation towards propionate ($P = 0.012$) at the expense of acetate ($P = 0.003$), as well as dramatically decreasing ammonia production (−77%; $P < 0.001$). Although total gas production was not affected by the inclusion of 2 g L^{-1} liquorice in the diet, methane production decreased ($P < 0.05$) by 35% (Table 3). Theoretical metabolic hydrogen production based on the VFA stoichiometry (Moss, Jouany and Newbold 2000) was also lower ($P = 0.002$) with 2 g L^{-1} of the flavonoid-rich extract.

The study of the fermentation pattern in the vessel over a 24 h period (days 17 and 18; Table S2, Supporting Information) showed the same differences between treatments as those described when studying the daily fermentation products in the overflow. Concentrations of D-, L- and total lactate were unaffected by the treatments. Sampling time had a strong effect on fermentation parameters with decreased ammonia concentration and increased propionate and butyrate concentrations after feeding ($P < 0.01$). D-, L- and total lactate concentrations decreased ($P < 0.001$) in samples taken after feeding.

Absolute and relative enzymatic activities (Table 4) increased ($P < 0.05$) when liquorice was added at 2 g L^{-1}. Sampling time also had an effect, with decreased xylanase and endoglucanase activities after feeding ($P < 0.01$). Quantitative PCR revealed decreases in only the relative abundance of anaerobic fungi ($P < 0.001$) with 2 g L^{-1} liquorice. Protozoa concentration decreased ($P < 0.001$) in vessels fed liquorice, with the highest dose having a stronger effect. The addition of liquorice, at all the doses tested, caused the elimination of the holotrich protozoa.

Bacterial 16S rRNA gene sequencing

Quality filtering resulted in 1 684 022 high-quality sequences (320 bp long) that clustered in 1811 different OTUs with 6195 reads per sample after normalisation.

Permutational analysis of variance (Table 5) showed a strong effect of both doses of liquorice on the structure of the bacterial community ($P = 0.0001$). However, no effect of time was observed ($P = 0.986$). Pairwise comparison showed that the structure of the bacterial community differed between control and liquorice treatments ($P = 0.0001$) and between liquorice 1 and liquorice 2 treatments ($P = 0.001$). This was confirmed by ANOSIM ($P = 0.001$), with the greatest differences found between control and liquorice 2 and liquorice 1 and liquorice 2 treatments.

To detect possible correlations between the structure of the bacterial community and rumen fermentation parameters, a dbRDA was performed. The primary axis accounted for 55.4% of the variation, and a clear separation by treatment was observed (Fig. 1). Ammonia and BCVFA concentrations in the vessel ($P < 0.001$) and bacterial richness ($P = 0.024$) were positively and negatively correlated to the structure of the bacterial community of control and liquorice 2 samples, respectively.

Regarding bacterial diversity (Table 6), the addition of liquorice decreased Shannon and Simpson indexes ($P = 0.001$ and $P < 0.001$, respectively) with the highest dose of liquorice having a stronger effect. Bacterial richness also decreased ($P < 0.001$) in the presence of liquorice extract as compared with the control.

No differences in bacterial abundances because of the addition of liquorice were observed at phylum (Table 7; $P > 0.1$) or family level (Table S3, Supporting Information; $P > 0.1$). At genera level (Table 8), only changes in less abundant genera were detected. The greatest change observed was the increased amount of Rikenella with liquorice as compared to the control treatment (P corrected value = 0.186).

Methanogens 16S rRNA gene sequencing

Quality filtering and removal of bacterial sequences resulted in 370 221 high-quality methanogen sequences (average length of 380 bp) that were clustered in to 33 unique OTUs with 3733 sequences per sample after normalisation.

Permutational analysis of variance (Table 5) showed an effect of liquorice addition on the structure of the archaeal community ($P = 0.0001$), but no effect of time was observed ($P = 0.993$). Pairwise comparison showed differences in the structure of the archaea community between control and liquorice treatments ($P = 0.0001$) and between liquorice 1 and liquorice 2 treatments ($P = 0.001$). ANOSIM analysis also showed these differences ($P = 0.001$), with the largest separation detected between the

Table 4. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g L^{-1}, respectively) and the sampling time (0, 2, 4 and 8 h after feeding) on rumen enzymatic activity and microbial numbers in the RUSITEC system.

	Treatment				P-Value	Time after feeding					P-Value	
	C	L1	L2	SED	Trt	0 h	2 h	4 h	8 h	SED	T	TrtxT
Absolute enzymatic activity (mmol of sugar gDM^{-1} min^{-1})												
Amylase	0.060[a]	0.069[a]	0.138[b]	0.016	0.005	0.083	0.097	0.092	0.083	0.011	0.435	0.464
Xylanase	0.081[a]	0.086[a]	0.103[b]	0.006	0.034	0.123[b]	0.083[a]	0.083[a]	0.071[a]	0.006	<0.001	0.166
Endoglucanase	0.062	0.067	0.074	0.004	0.097	0.085[b]	0.064[a]	0.062[a]	0.060[a]	0.003	<0.001	0.031
Relative enzymatic activity (mmol of sugar $gProtein^{-1}$ min^{-1})												
Amylase	0.306[a]	0.315[a]	0.664[b]	0.031	<0.001	0.405	0.444	0.401	0.463	0.061	0.567	0.391
Xylanase	0.42[a]	0.396[a]	0.493[b]	0.022	0.012	0.606[b]	0.392[a]	0.357[a]	0.390[a]	0.039	<0.001	0.437
Endoglucanase	0.326[a]	0.314[a]	0.356[b]	0.010	0.013	0.421[b]	0.304[a]	0.267[a]	0.337[a]	0.030	0.006	0.940
Microbial numbers												
Bacteria (log copies gDM^{-1})	11.4	11.4	11.4	0.033	0.093	11.3	11.4	11.4	11.4	0.029	0.068	0.135
Methanogens (log copies gDM^{-1})	9.66[a]	9.82[b]	9.61[a]	0.051	0.016	9.83[c]	9.73[b]	9.62[a]	9.62[a]	0.032	<0.001	0.182
Anaerobic fungi (log copies gDM^{-1})	7.99[b]	7.47[b]	4.71[a]	0.277	<0.001	7.04	6.75	6.69	6.41	0.250	0.160	0.690
Protozoa (log cells mL^{-1})												
Total	3.57[c]	2.89[b]	2.30[a]	0.114	<0.001							
Holotrichs	2.91[b]	0[a]	0[a]	0.062	<0.001							
Entodinomorphs	3.46[c]	2.89[b]	2.30[a]	0.126	<0.001							

[a–c]Means with different superscript differ ($P < 0.05$). Trt: treatment; T: time.

Table 5. Effects of the supplementation with liquorice extract (1 and 2 g L^{-1}) on the structure of the bacterial communities in the rumen simulating fermenter RUSITEC.

	Bacteria		Archaea	
	P(MC) (Per-manova)	R-value (ANOSIM)	P(MC) (Per-manova)	R-value (ANOSIM)
Treatment effect	0.0001	0.981	0.0001	0.744
Pairwise comparison				
Control vs Liquorice 1	0.0001	0.924	0.0001	0.573
Control vs Liquorice 2	0.0001	1	0.0001	0.971
Liquorice 1 vs liquorice 2	0.0001	1	0.0001	0.867
Time effect	0.9863	−0.161	0.993	−0.194
Pairwise comparison				
t0 vs t2	0.9179	−0.163	0.943	−0.208
t0 vs t4	0.82	−0.149	0.948	−0.125
t0 vs t8	0.4681	−0.087	0.578	−0.146
t2 vs t4	0.9986	−0.26	0.999	−0.240
t2 vs t8	0.8258	−0.167	0.935	−0.212
t4 vs t8	0.9309	−0.146	0.891	−0.222

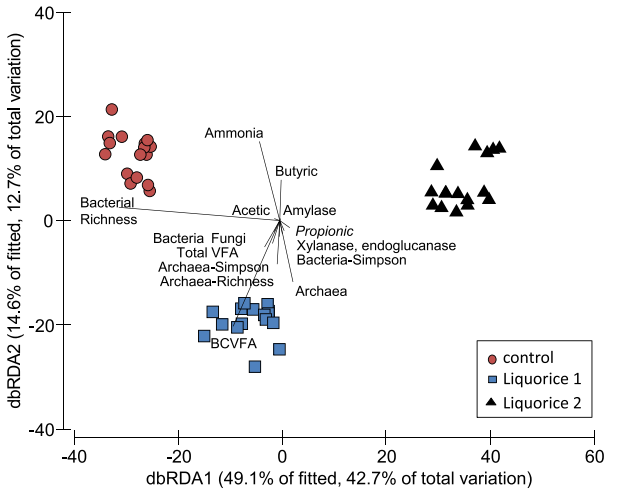

Figure 1. DbRDA illustrating the relationship between the structure of the bacterial community (based on Bray–Curtis distance matrices calculated of normalised and transformed abundance data) with the rumen fermentation pattern and microbial numbers and diversity in the RUSITEC system.

archaeal communities corresponding to control and liquorice 2 treatments. dbRDA (Fig. 2) primary axes displayed 71.3% of the variation and a separation between treatments. Several variables (total and molar proportions of VFA, ammonia and archaea diversity and richness) were positively correlated ($P < 0.001$) with the structure of the archaeal population in vessels corresponding to the liquorice 2 treatment.

Contrary to the effects on bacterial community structure, methanogens richness and diversity was unaffected by the addition of liquorice (Table 6; $P > 0.05$). Based on the RIM-DB database, three families (Methanomassiliicoccaceae, Methanosarcinaceae and Methanobacteriaceae) made up the archaeal population in this experiment. The addition of liquorice extract influenced the abundance of the main methanogen groups with the highest dose having a stronger effect (Table 9). When added at 2 g L^{-1}, the flavonoid-rich extract dramatically decreased Methanomassiliicoccus Group 12 ($P = 0.035$) and it also reduced Group 3a ($P = 0.092$). On the contrary, the highest dose of liquorice promoted an increased in the abundance of Methanomassiliicoccus Group 10 ($P = 0.035$), Methanobrevibacter ($P = 0.092$) and Methanosphaera ($P = 0.053$).

DISCUSSION

Flavonoids have received interest as promising alternatives to antibiotics in ruminant feeding because of their antimicrobial activity (Cheng *et al.* 2014). Indeed, *in vitro* studies have shown that flavonoid-rich plant extracts reduce methane production in the rumen (Bodas *et al.* 2008; Patra and Saxena 2010; Oskoueian, Abdullah and Oskoueian 2013) that has been associated with its effect on the methanogen (Patra and Saxena 2010) and

Table 6. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g L^{-1}, respectively) and the sampling time (0, 2, 4 and 8 h after feeding) on the structure of the bacteria and methanogen communities in the RUSITEC system.

	Treatment				P-Value	Time after feeding					P-Value	
	C	L1	L2	SED	Trt	0 h	2 h	4 h	8 h	SED	T	TrtxT
Bacteria												
Richness	497[c]	387[b]	231[a]	23.8	<0.001	387	369	387	343	12.6	0.018	0.160
Simpson index	0.959[b]	0.944[b]	0.923[a]	0.008	0.010	0.942	0.944	0.952	0.931	0.006	0.039	0.577
Shannon index	4.35[c]	3.95[b]	3.48[a]	0.115	<0.001	3.98	3.94	4.04	3.76	0.067	0.012	0.454
Archaea												
Richness	18.9	20.8	20.3	0.885	0.155	19.0	19.6	21.1	20.3	0.616	0.042	0.550
Simpson index	0.525	0.725	0.645	0.090	0.162	0.648	0.629	0.637	0.612	0.021	0.369	0.516
Shannon index	1.26	1.77	1.49	0.212	0.135	1.54	1.51	1.53	1.45	0.053	0.296	0.315

[a–c]Means with different superscript differ ($P < 0.05$). Trt: treatment; T: time.

Table 7. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g L^{-1}, respectively) and the sampling time (0, 2, 4 and 8 h after feeding) on relative abundance of bacteria phyla present at an average of more than 0.5% (false discovery rate for Benjamini–Hochberg: 0.25).

	Treatment			Time				SED			Uncorrected P			Benjamini–Hochberg P-Value		
	C	L1	L2	T0	T2	T4	T8	Trt	T	TrtxT	Trt	T	TrtxT	Trt	T	TrtxT
Proteobacteria	0.181	0.218	0.179	0.200	0.189	0.166	0.217	0.034	0.020	0.045	0.478	0.118	0.307	0.598	0.393	0.512
Bacteroidetes	0.482	0.473	0.517	0.470	0.496	0.502	0.495	0.032	0.029	0.054	0.406	0.623	0.472	0.580	0.811	0.590
Firmicutes	0.257	0.257	0.259	0.265	0.256	0.268	0.242	0.043	0.025	0.057	0.999	0.629	0.263	0.999	0.811	0.512
Spirochaetes	0.021	0.017	0.015	0.021	0.019	0.020	0.011	0.003	0.003	0.005	0.213	0.029	0.213	0.456	0.145	0.512
unclassified	0.021	0.009	0.007	0.013	0.011	0.014	0.012	0.007	0.003	0.009	0.217	0.649	0.251	0.456	0.811	0.512
Tenericutes	0.012	0.013	0.012	0.013	0.013	0.013	0.010	0.003	0.003	0.005	0.900	0.580	0.359	0.999	0.811	0.513
Verrucomicrobia	0.012	0.001	0.000	0.004	0.004	0.005	0.005	0.004	0.003	0.006	0.049	0.813	0.905	0.430	0.813	0.905
Fibrobacteres	0.006	0.003	0.001	0.005	0.004	0.003	0.001	0.002	0.001	0.002	0.086	0.020	0.104	0.430	0.145	0.512
Synergistetes	0.001	0.004	0.006	0.003	0.004	0.005	0.002	0.002	0.001	0.003	0.228	0.389	0.627	0.456	0.811	0.697
Elusimicrobia	0.006	0.003	0.001	0.004	0.003	0.003	0.003	0.003	0.002	0.004	0.356	0.746	0.047	0.580	0.813	0.470

Trt: treatment; T:time.

protozoal populations (Kim *et al.* 2015). Furthermore, recent *in vivo* studies have reported changes in the bacteria community as a consequence of supplementing the diet with flavonoids (de Nardi *et al.* 2016; Kasparovska *et al.* 2016; Zhan *et al.* 2017).

Liquorice, the root of the *Glycyrrhiza* species, has long been used worldwide in herbal medicine and as a natural sweetener (Asl and Hosseinzadeh 2008; Damle 2014). More than 20 triterpenoids and nearly 300 flavonoids have been isolated from liquorice (Wang *et al.* 2015). Glycyrrhizin, a triterpenoid saponin, is considered as the bioactive constituent of liquorice (Asl and Hosseinzadeh 2008). However, it has been shown that many biological activities of liquorice, including estrogenic, anti-cancer, anti-microbial, skin whitening and metabolic syndrome preventive, could be ascribed to its isoflavonoid constituents (Vaya, Belinky and Aviram 1997). Isoflavonoids (3-phenyl benzopyrans) differ from other classes of flavonoids due to their greater structural variability, their presence mainly in free form, rather than as a glycoside, and by the greater frequency of isoprenoid substitution (Munke, Viswanathan and Phadatare 2011).

Our results showed that liquorice extract had a strong antiprotozoal effect *in vitro* when measuring protozoal activity based on the amount of released [^{14}C] from labelled bacteria. Incubations for 24 h revealed that doses of 1 and 2 g L^{-1} decreased ammonia concentration by 11% and 21% and increased propionate molar proportion by 14% and 32%, respectively, without impairing the overall fermentation process. The highest dose of liquorice tested also decreased butyrate molar proportions by 21%. Stoichiometrically, and based on the equation of Moss, Jouany and Newbold (2000), the shift in the fermentation pattern observed with 1 and 2 g L^{-1} of liquorice extract should have resulted in a reduction in methane of 3% and 13%, respectively. When these doses of isoflavonoid-rich extract were tested for their long-term effects in the RUSITEC system, similar effects on fermentation, without negative effects on nutrient digestibility were observed. The addition of 2 g L^{-1} of the extract also caused a decrease in methane per gram of disappeared OM (−27%). A decrease in total VFA and a substantial shift in the fermentation pattern from acetate towards propionate was observed, leading to a decrease in the theoretical metabolic hydrogen production (−13%).

Despite the inherent difficulty of maintaining high numbers of protozoa in the RUSITEC system (Hillman, Williams and Lloyd 1991), protozoal numbers in our study were reasonable (3775 cells mL^{-1} for the control), allowing the assessment of the effect of the treatments on the protozoal community. The effects of our liquorice extract on methane emission could have been associated with a decreased protozoa population (−79% and −94% for doses of 1 and 2 g L^{-1}, respectively) since protozoa provide hydrogen as a reducing substrate to methanogens (Newbold *et al.* 2015). The elimination of holotrich protozoa, which play a disproportionate role in supporting methanogenesis (Newbold *et al.* 2015), would be in line with the reduction in methane reported. Although 2 g L^{-1} of liquorice also caused a great reduction in

Table 8. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g L^{-1}, respectively) and the sampling time (0, 2, 4 and 8 h after feeding) on relative abundance of bacteria genera present at an average of more than 0.2% (false discovery rate for Benjamini–Hochberg: 0.25).

	Treatment			Time				SED			Uncorrected *P*			Benjamini-Hochberg *P*-Value		
	C	L1	L2	T0	T2	T4	T8	Trt	T	TrtxT	Trt	T	TrtxT	Trt	T	TrtxT
Ruminobacter	0.054	0.055	0.035	0.035	0.035	0.047	0.076	0.016	0.027	0.043	0.400	0.355	0.764	0.777	0.880	0.882
Prevotella	0.250	0.274	0.277	0.264	0.279	0.280	0.246	0.035	0.030	0.057	0.721	0.606	0.343	0.901	0.880	0.828
Unclassified	0.226	0.203	0.192	0.216	0.198	0.219	0.195	0.018	0.023	0.039	0.237	0.551	0.205	0.631	0.880	0.828
Christensenella	0.018	0.012	0.017	0.014	0.011	0.016	0.023	0.007	0.006	0.011	0.624	0.245	0.273	0.901	0.880	0.828
Anaerovorax	0.021	0.004	0.012	0.013	0.013	0.012	0.013	0.009	0.011	0.018	0.228	0.991	0.401	0.631	0.991	0.828
Vampirovibrio	0.002	0.009	0.003	0.003	0.004	0.005	0.007	0.002	0.004	0.006	0.029	0.683	0.882	0.186	0.880	0.882
Selenomonas	0.010	0.017	0.014	0.013	0.019	0.011	0.012	0.005	0.004	0.008	0.467	0.196	0.539	0.830	0.880	0.828
Roseburia	0.003	0.006	0.003	0.005	0.006	0.003	0.002	0.004	0.003	0.007	0.731	0.600	0.401	0.901	0.880	0.828
Paraprevotella	0.042	0.044	0.039	0.057	0.029	0.050	0.029	0.024	0.025	0.045	0.975	0.547	0.570	0.975	0.880	0.828
Treponema	0.011	0.012	0.012	0.014	0.013	0.011	0.010	0.003	0.003	0.005	0.937	0.481	0.222	0.975	0.880	0.828
Anaeroplasma	0.007	0.010	0.011	0.009	0.010	0.009	0.009	0.002	0.002	0.004	0.241	0.890	0.602	0.631	0.949	0.828
Subdivision5_genera_incertae_sedis	0.009	0.002	0.001	0.006	0.005	0.005	0.002	0.005	0.003	0.007	0.276	0.529	0.792	0.631	0.880	0.882
Fibrobacter	0.005	0.003	0.002	0.006	0.004	0.003	0.002	0.002	0.001	0.003	0.413	0.059	0.156	0.777	0.880	0.828
Acidaminococcus	0.019	0.013	0.018	0.022	0.016	0.016	0.013	0.001	0.004	0.006	0.008	0.163	0.799	0.186	0.880	0.882
Solobacterium	0.002	0.003	0.002	0.002	0.003	0.002	0.002	0.000	0.001	0.001	0.259	0.604	0.864	0.631	0.880	0.882
Pyramidobacter	0.003	0.003	0.004	0.003	0.005	0.003	0.003	0.002	0.002	0.003	0.970	0.427	0.670	0.975	0.880	0.833
Anaerovibrio	0.020	0.017	0.017	0.015	0.020	0.017	0.021	0.008	0.007	0.014	0.919	0.690	0.198	0.975	0.880	0.828
Streptococcus	0.057	0.033	0.040	0.044	0.038	0.043	0.048	0.010	0.016	0.026	0.111	0.887	0.433	0.507	0.949	0.828
Pseudobutyrivibrio	0.015	0.017	0.018	0.017	0.018	0.015	0.016	0.006	0.007	0.012	0.856	0.955	0.356	0.975	0.986	0.828
Succinivibrio	0.121	0.145	0.139	0.134	0.152	0.118	0.137	0.027	0.026	0.048	0.670	0.603	0.252	0.901	0.880	0.828
Succiniclasticum	0.021	0.025	0.024	0.020	0.026	0.024	0.021	0.005	0.009	0.014	0.732	0.852	0.621	0.901	0.949	0.828
Coprococcus	0.003	0.005	0.006	0.004	0.005	0.005	0.005	0.001	0.001	0.002	0.142	0.285	0.323	0.568	0.880	0.828
Phocaeicola	0.009	0.004	0.006	0.007	0.005	0.006	0.008	0.001	0.003	0.005	0.044	0.692	0.582	0.235	0.880	0.828
Lactobacillus	0.014	0.017	0.016	0.019	0.013	0.012	0.019	0.003	0.005	0.008	0.552	0.392	0.436	0.901	0.880	0.828
Rikenella	0.012	0.022	0.021	0.013	0.019	0.019	0.022	0.003	0.005	0.009	0.027	0.341	0.336	0.186	0.880	0.828
Sphaerochaeta	0.004	0.004	0.006	0.005	0.005	0.005	0.004	0.001	0.001	0.002	0.396	0.539	0.879	0.777	0.880	0.882
Asteroleplasma	0.002	0.002	0.003	0.002	0.003	0.003	0.002	0.001	0.001	0.002	0.583	0.884	0.677	0.901	0.949	0.833
Candidatus Endomicrobium	0.001	0.003	0.005	0.002	0.003	0.004	0.004	0.002	0.002	0.004	0.188	0.715	0.599	0.631	0.880	0.828
Eubacterium	0.003	0.004	0.004	0.004	0.004	0.003	0.003	0.000	0.001	0.001	0.023	0.054	0.290	0.186	0.880	0.828
Butyricimonas	0.002	0.003	0.003	0.002	0.003	0.002	0.002	0.001	0.001	0.002	0.631	0.488	0.573	0.901	0.880	0.828
Mucinivorans	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.001	0.001	0.002	0.786	0.629	0.409	0.932	0.880	0.828
Anaerocella	0.012	0.009	0.028	0.012	0.016	0.014	0.023	0.005	0.009	0.015	0.018	0.604	0.501	0.186	0.880	0.828

Trt: treatment; T:time.

Table 9. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g L^{-1}, respectively) and the sampling time (0, 2, 4 and 8 h after feeding) on relative abundance of archaea genera present at an average of more than 0.2% (false discovery rate for Benjamini–Hochberg: 0.25).

	Treatment			Time				SED			Uncorrected *P*			Benjamini-Hochberg *P*-Value		
	C	L1	L2	T0	T2	T4	T8	Trt	T	TrtxT	Trt	T	TrtxT	Trt	T	TrtxT
Methanomassiliicoccus G12	0.588	0.171	0.009	0.231	0.256	0.269	0.268	0.109	0.012	0.111	0.005	0.034	0.327	0.035	0.170	0.483
Methanomassiliicoccus G9	0.222	0.423	0.258	0.260	0.310	0.299	0.335	0.164	0.026	0.168	0.470	0.114	0.337	0.470	0.380	0.483
Methanomassiliicoccus G10	0.002	0.018	0.309	0.126	0.115	0.112	0.086	0.068	0.011	0.070	0.007	0.017	<0.001	0.035	0.170	< 0.011
Methanomassiliicoccus G11	0.023	0.099	0.001	0.042	0.043	0.041	0.038	0.057	0.004	0.057	0.267	0.522	0.452	0.297	0.522	0.483
Methanomassiliicoccus G3a	0.039	0.012	0.001	0.013	0.018	0.018	0.019	0.012	0.003	0.012	0.042	0.252	0.140	0.092	0.407	0.483
Methanomassiliicoccus G3b	0.017	0.024	0.001	0.020	0.011	0.013	0.012	0.011	0.005	0.013	0.180	0.260	0.483	0.225	0.407	0.483
Methanomassiliicoccus G8	0.023	0.056	0.004	0.041	0.024	0.021	0.024	0.017	0.013	0.026	0.062	0.326	0.464	0.103	0.407	0.483
Methanimicrococcus	0.002	0.040	0.004	0.023	0.014	0.014	0.011	0.020	0.009	0.024	0.176	0.425	0.402	0.225	0.472	0.483
Methanobrevibacter	0.083	0.151	0.354	0.218	0.186	0.192	0.189	0.086	0.019	0.091	0.046	0.324	0.112	0.092	0.398	0.466
Methanosphaera	0.0004	0.006	0.059	0.025	0.021	0.021	0.020	0.015	0.002	0.016	0.016	0.155	0.482	0.053	0.388	0.483

Trt: treatment; T:time.

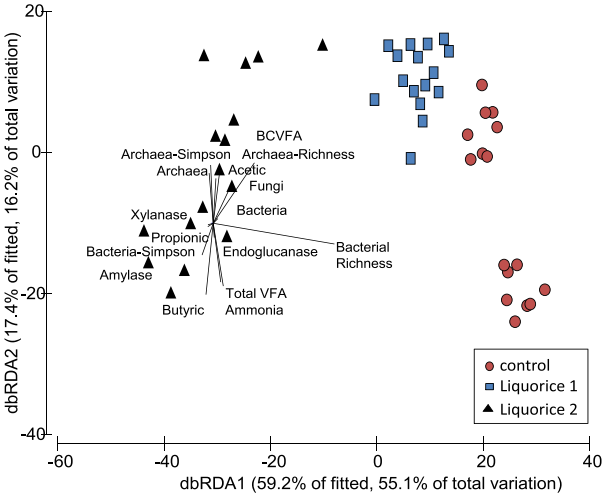

Figure 2. DbRDA illustrating the relationship between the structure of the archaeal community (based on Bray–Curtis distance matrices calculated of normalised and transformed abundance data) with the rumen fermentation pattern and microbial numbers and diversity in the RUSITEC system.

anaerobic fungi, which together with protozoa plays a significant role in the degradation of ingested plant cellulosic fibres, the digestibility of the fibre resulted unaffected. Possibly an increase in bacterial activity, as reflected in a greater xylanase activity with liquorice, might have compensated for the decrease in protozoal and fungal activity. The greatest effect observed in the presence of liquorice was the reduction in ammonia production (−51 and −77% with 1 and 2 g L^{-1} of liquorice, respectively) that could have also been related to the decrease in protozoa as they are involved in the turnover of bacterial protein due to their predatory activity (Newbold *et al.* 2015). It is also possible that the formation of isoflavonoids-protein complexes could have reduced the availability of nitrogen to rumen microorganisms, as has been previously reported for other polyphenolic compounds (Ozdal, Capanoglu and Altay 2013). Isoflavonoids may also have other effects on rumen fermentation and microbial activities: some authors have suggested that derivatives from the microbial degradation of flavonoids can be used as alternative carbon source for rumen microbial activities (McSweeney *et al.* 2001; Smith, Zoetendal and Mackie 2005; Ouskoueian *et al.* 2013), whilst others have proposed that flavonoids could act as a hydrogen sink via cleavage of ring structures and reductive dihydroxylation (Becker *et al.* 2013).

Although the total number of bacteria were unaffected by the addition of liquorice, the isoflavonoid-rich extract promoted a less diverse bacterial community. ANOSIM analysis showed that the bacterial community structure was highly separated between treatments. Only changes in the relative abundance of less abundant genera were however observed. The greatest change was observed for Rikenella that are thought to be involved in structural carbohydrates degradation (Pitta *et al.* 2010). Its increase in the presence of liquorice would be in line with the observed increase in xylanase activity. Contrary to previous studies (Oskoueian, Abdullah and Oskoueian 2013; Seradj *et al.* 2014), no major effects on archaea numbers were observed with the addition of liquorice extract. The isoflavonoid-rich extract did not significantly affect archaea diversity. Liquorice extract had an effect on the structure of the methanogen community that differed between treatments, although not to the same extent as that of the bacterial communities. A shift in the methanogen community towards one less effective in producing methane could be suggested to explain differences in methane emissions. Although it has been reported that methane emission can be related to the concentration of archaea in rumen digesta (Wallace *et al.* 2014), it seems that it is the metabolic activity of individual species rather than the number of archaea what is essential for the level of methane production (Shi *et al.* 2014). Methanomassiliicoccus Group12 and Group 3a were replaced by Methanomassiliicoccus Group 10, Methanosphaera and Methanobrevibacter. Methanobrevibacter, theoretically less active in methane production (Kang *et al.* 2013) increased by 0.27 log units with 2 g L^{-1} of the extract, as compared to the control. This observation was also reported by Belanche *et al.* (2016) when using ivy saponins in RUSITEC.

Liquorice extract added at 1 g L^{-1} decreased ammonia production without affecting the overall fermentation process. When added at 2 g L^{-1}, decreases in not only ammonia production but also methane and total VFA production were observed. These effects in fermentation were probably related to decreases in protozoa numbers, a less diverse bacteria population as well as changes in the structure of both bacteria and archaea communities. The inclusion of an isoflavonoid-rich extract from liquorice in the diet could potentially improve the efficiency of the feed utilisation by ruminants. While we speculate that the observed effects could be attributed to the high content of isoflavonoids, and particularly glabridin, the contribution of other phytochemical to the reported effects cannot be ruled out.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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